POSSIBLE PROTECTIVE EFFECT OF GINGER ON THE BUCCAL MUCOSA OF METHOTREXATE TREATED MALE ALBINO RATS (MOLECULAR AND HISTOLOGICAL EVALUATION)

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ABSTRACT

Introduction: Methotrexate (MTX) is widely used as a chemotherapeutic agent. However, severe side effects and toxic conditions limit its efficacy. Recently, it has been focused on the protective effect of plant derived natural compounds, such as ginger, against chemicals inducing toxicity.

Aim: The aim of the present study was to elucidate the possible protective effect of ginger administration on the buccal mucosa of rats under MTX treatment.

Material & methods: Eighteen adult male albino rats were divided into three groups (six animals each). **Group I (Control group):** rats were sacrificed at the end of the experimental design. **Group II (MTX group):** received 15mg/Kg/day of MTX for 3 successive days. **Group III (MTX and ginger group):** received ginger (200mg/kg/day) for 15 successive days then, MTX as in group II concomitantly with ginger administration daily. Rats of groups II and III were sacrificed immediately after the last dose of MTX. Specimens were prepared for histological examination and molecular study.

Results: Histopathological examination of group II revealed atrophy and complete loss of normal architecture with cellular deformities within the buccal mucosal cells. These changes were markedly alleviated in group III specimens. Molecular results supported the histological results which presented marked decrease in DNA relative concentration in group II when compared to the control one, while less obvious decrease in DNA relative concentration was detected in group III.

Conclusion: Pre and co administration of ginger protected and ameliorated most of the damage caused by MTX in the buccal mucosa structure and DNA.

KEY WORDS: Buccal mucosa, Methotrexate, Ginger, DNA.

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INTRODUCTION

Methotrexate (MTX), a folate antagonist agent, is a potent cytotoxic drug. It has been used as a chemotherapeutic agent for treatment of multiple disorders including acute lymphoblastic leukemia, non-Hodgkin's lymphoma, breast cancer and testicular tumors (**Jensen et al, 2008**) [1]. It has also been found to have a vital therapeutic role as an anti-inflammatory and immunosuppressive agent (**Abd-Allah and Sharaf El-Din, 2013 & Dalaklioglu et al, 2013**) [2,3]. MTX is well absorbed from the gastrointestinal tract and is then distributed in tissues, body fluids and also crosses the blood-brain barrier (**Imani and Ainehchi, 2014**) [4].

However, severe side effects and toxic conditions limit the efficacy of MTX. Gastrointestinal toxicity is a main factor limiting its use (**Abd-Allah and Sharaf El-Din, 2013**) [2]. Moreover, growth retardation, renal insufficiency, stomatitis, marrow suppression and hepatic failure were also documented (**Cohen, 2007**) [5]. Secondary or systemic infections may result from major side effects of MTX, which lead to discontinuation of the treatment or postponing the next cycle of chemotherapy (**Chen et al, 2013**) [6]. MTX was also reported to interfere with DNA synthesis and to cause its destruction (**Saxena et al, 2009**) [7].

Chemotherapeutic drugs are well identified to damage both rapidly and slowly dividing cells in the body. Normal host cells can also be affected as in radiotherapy. Commonly affected cells are those from the gastrointestinal epithelium, including cells of the buccal mucosa and bone marrow cells (**Munaretto et al, 2011**) [8].

MTX is well known to cause increased production of reactive oxygen species (ROS) resulting in increased oxidative stress (**Miyazono et al, 2004**) [9]. Based on this result, several studies showed positive effects by using antioxidant agents in order to avoid MTX- induced damage (**Van'tland et al, 2004 and Ciralik et al, 2006**) [10,11].

Recently, it has been focused on natural products and their active ingredients as sources for new drug therapy. Herbs and spices are used nowadays to treat several diseases in a safe way (**Ali, 2011**) [12]. Plant derived natural compounds were found to have a protecting effect against chemicals toxicity (**Delmanto et al, 2001**) [13].

Ginger (Zingiber officinale Roscoe) has been used as a spice for many years ago, and it is utilized today for treatment of various diseases (**Abd-Allah and Sharaf El-Din, 2013**) [2]. Ginger is widely used to relief symptoms of nausea and vomiting accompanying motion sickness, surgical treatments and pregnancy (**Gilani and Rahman, 2005**) [14]. Moreover, ginger extracts are rich in shagaols and gingerols which show anti-inflammatory and anti-oxidant properties (**Attyah, and Ismail, 2012**) [15]. It was reported that ginger is a potent and non-toxic oral medication with undetectable side effects (**Ali, 2011**) [12].

From the previously mentioned data, the present study aimed to elucidate the possible protective effect of ginger administration on the buccal mucosa of rats under methotrexate treatment.

MATERIAL AND METHODS

Animals

Eighteen adult male Swiss Albino rats weighing 150-200 gm were used in the present work. They were allowed to access freely standard balanced diet and freshwater supply.

Experimental design

The animals were divided into three groups (six animals each) and were treated as follow:

Group I (Control group): Included 6 rats in which 3 received intraperitoneal injection of 1ml/Kg dose of sterile saline (0.9% NaCl) daily which is the vehicle of MTX. The other 3 rats received 1ml of 0.5% carboxymethylcellulose (CMC) in water daily (the vehicle of ginger) throughout the experimental period using gastric tube. Rats were sacrificed at the end of the experimental design.

Group II (MTX group): Included 6 rats that received intraperitoneal injection of 15mg/Kg/day methotrexate drug (Orion Corporation Espoo, Finland) dissolved in 0.95 NaCl for 3 successive days **(Mcbride et al, 1987)** [16].

Group III (MTX and ginger group): Included 6 rats that were given oral dose of ginger (200mg/kg/day) (Sigma) suspended in 0.5% CMC delivered by gastric tube **(Abd-Allah and Sharaf El-Din, 2013)** [2] for 15 successive days before MTX injection. Then rats received MTX as in group II concomitantly with ginger administration daily.

Rats of groups II and III were sacrificed immediately after the last dose of MTX by ketamine over dose.

At the end of the experimental period, the buccal mucosa was carefully dissected from all animals and washed immediately under running water to remove any debris and blood. Each specimen was divided into 2 halves to be prepared for routine histological examination and molecular study.

Light microscopic examination

The first half was fixed in 10% neutral formalin for 48 hours and embedded in paraffin. Histological sections of 5 μm thickness were obtained and stained with haematoxylin and eosin stain.

Molecular study

The second half was kept frozen for molecular study.

Molecular method:

Genomic DNA purification from buccal tissue using the Gentra Puregene Tissue Kit: (Williams et al., 1990) [17]

Procedure:

1. Dissect buccal tissue sample quickly and freeze in liquid nitrogen. Grind 50–100 mg frozen buccal tissue in liquid nitrogen with a mortar and pestle. Work quickly and keep tissue on ice at all times, including when tissue is being weighed.

2. Dispense 3 ml Cell Lysis Solution into a 15 ml grinder tube on ice, and add the ground tissue from the previous step. Complete cell lysis by following step 2a or 2b below:

   2a. Heat at 65°C for 15 min to 1 h.

   2b. If maximum yield is required, add 15 μl Puregene Proteinase K, mix by inverting 25 times, and incubate at 55°C for 3 h. or until tissue has completely lysed. Invert tube periodically during the incubation. The sample can be incubated at 55°C overnight for maximum yields.

3. Add 15 μl RNase A Solution, and mix the sample by inverting 25 times. Incubate at 37°C for 15–60 min.

4. Incubate for 3 min on ice to quickly cool the sample.

5. Add 1 ml Protein Precipitation Solution, and vortex vigorously for 20 s. at high speed.

6. Centrifuge for 3 min at 13,000–16,000 x g. The precipitated proteins should form a tight pellet. If the protein pellet is not tight, incubate on ice for 5 min and repeat the centrifugation.

7. Pipet 3 ml isopropanol into a clean 15 ml centrifuge tube and add the supernatant from the previous step by pouring carefully. Be sure the protein pellet is not dislodged during pouring.

   Note: If the DNA yield is expected to be low (<1 μg) add 0.5 μl Glycogen Solution (cat. no. 158930).

8. Mix by inverting gently 50 times.

9. Centrifuge for 3 min at 2000 x g.

10. Carefully discard the supernatant, and drain the tube by inverting on a clean piece of absorbent paper, taking care that the pellet remains in the tube.

11. Add 3 ml of 70% ethanol and invert several times to wash the DNA pellet.

12. Centrifuge for 1 min at 2000 x g.

13. Carefully discard the supernatant. Drain the tube on a clean piece of absorbent paper, taking care that the pellet remains in the tube. Allow to air dry for up to15 min. The pellet might be loose and easily dislodged.

14. Add 150 μl DNA Hydration Solution and vortex for 5 s at medium speed to mix.

15. Incubate at 65°C for 1 h to dissolve the DNA.

16. Incubate at room temperature overnight with gentle shaking. Ensure tube cap is tightly closed to avoid leakage. Samples can then be centrifuged briefly and transferred to a storage tube.

Inter Simple Sequence Repeats (ISSRs):

ISSR markers involve PCR amplification of DNA using a single primer composed of a microsatellite sequence such as (GA)8 anchored at the 3´ or 5´ end by 2 – 4 arbitrary, often degenerate, nucleotides. The sequences of repeats and anchored nucleotides are randomly selected. The technique was carried out according to **Adawy et al, 2004** [18]. Ten oligonucleotides composed wholly of defined, short tandem repeat sequences with anchor, and representing different microsatellites (di- and tri-repeats) have been used as generic primers in PCR amplification of inter simple sequence repeat regions.

ISSR-PCR Reaction and Thermocycling Profile

PCR was performed in 25 ul reaction volume containing 1X PCR buffer, 1.75 mM MgCl2, 5 mM of each dNTPs, 40 pM oligonucleotide primer, 25ng genomic DNA and 1 Unit of Taq DNA polymerase. A high stringency touchdown and hot start thermocycling profile was used. This was performed to avoid any mismatch between the primer and the template as follows: an initial denaturation step for 5 min at 94ºC followed by 40 cycles at 94ºC for 30 second, 65ºC for 45 second

and 72ºC for 1min and an extension cycle at 72ºC for 7 min. The amplification products were resolved by electrophoresis on 1.5% agarose gel containing ethidium bromide (0.5μg/ml) in 1X TBE buffer. A 100bp DNA ladder was used as a molecular size standard. PCR products were visualized on UV light and photographed using a Gel Documentation System (BIO -RAD).

Primers

Six primers were used in this study to amplify the genomic DNA. The sequence of the primers is demonstrated in table 1.

TABLE (1): Code and sequence of the primers used in ISSR detection.

Primer Code	Sequence
IS1	TAT(CA)$_7$C
IS2	CAC(TCC)$_5$
IS3	TTT(TCC)$_5$
IS4	CAT(CA)$_7$T
IS5	(GA)$_8$CG
IS6	ATTA(CA)$_7$

RESULTS

H&E results

Control group (group I)

The buccal mucosa of the control group demonstrated keratinized stratified squamous epithelial covering with uniform arrangement of epithelial strata. The basal cells had oval nuclei. The prickle cell layer consisted of numerous polyhedral cells, while those of the granular layer appeared flattened with numerous basophilic keratohyaline granules. The overlying keratinous layer was thick and exhibited eosinophilic stain. Moreover, the rete pegs showed short and broad folding while the underlying connective tissue (C.T.) revealed numerous interlacing collagen fibers and scattered fibroblasts (Fig. 1)
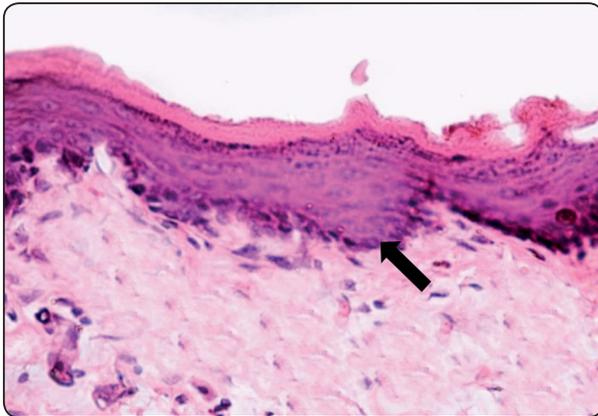
FIG. (1) Photomicrograph of rat buccal mucosa of control group showing normal layers of keratinized stratified squamous epithelium & underlying C.T., short & broad epithelial rete pegs (arrow) (H&E: 400X)

MTX treated group (group II)

Histologic sections from MTX treated rats revealed apparent thinning and atrophy of the epithelial lining with areas of desquamation and denudation of the epithelium and overlying keratin (Fig. 2;a). Complete absence of the keratinous layer was detected in some areas (Fig. 2;b). The epithelial layers demonstrated complete loss of normal architecture with cellular deformities. The basal cells appeared severely destructed, pleomorphic and lacked their normal arrangement on the basement membrane. Wide spread intracellular vacuolaization was markedly spotted within the epithelial cells. Some specimens showed areas of severely deformed epithelial cells invading the underlying connective tissue (Figs. 2;b,c). Apoptotic cells were also detected showing pyknosis, nuclear fragmentation
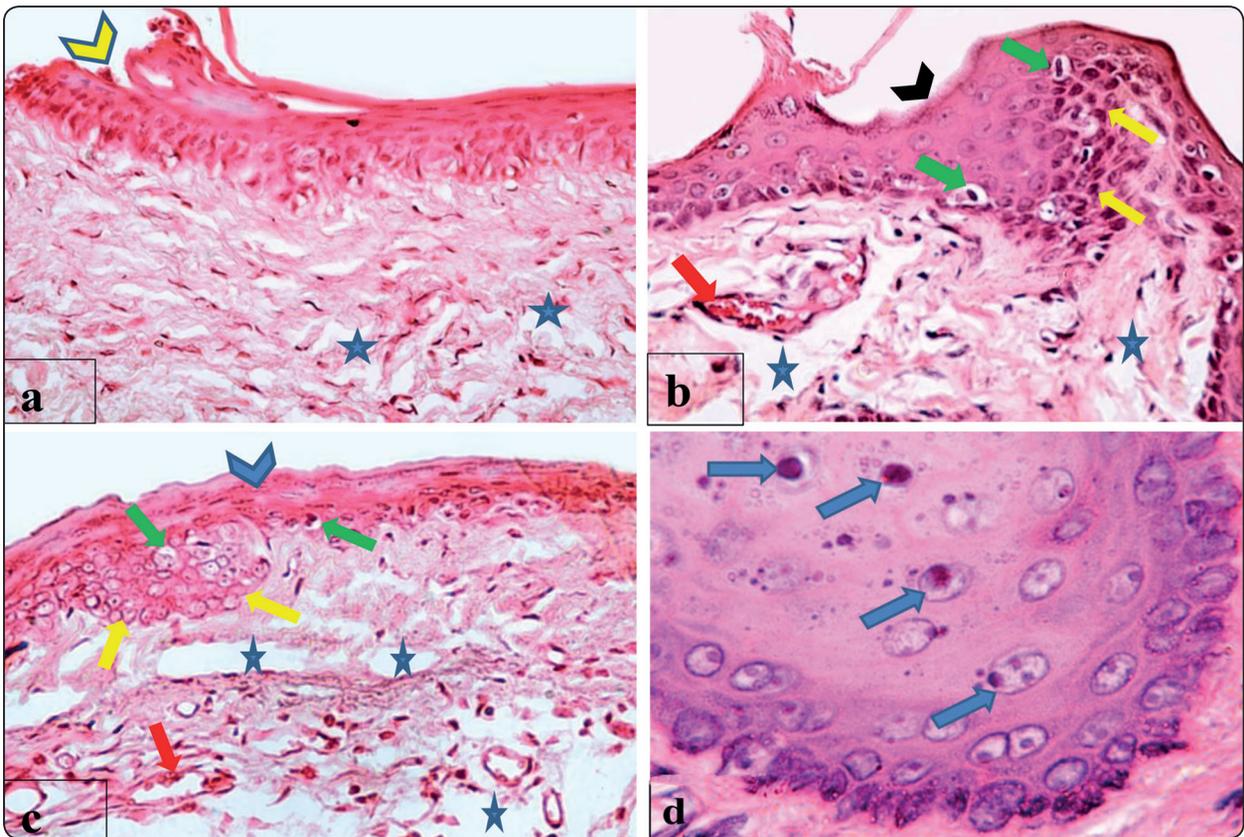

FIG. (2) Photomicrograph of rat buccal mucosa of group II showing: atrophy of the epithelial lining (2;a,b,c), areas of desquamation of the epithelium and keratin (yellow arrow head) (2;a), absence of the keratinous layer (black arrow head) (2;b), deformed & pleomorphic basal cells invading the underlying C.T. (yellow arrows), intracellular vacuolaization (green arrows) (2;b,c), apoptotic cells (blue arrows) (2;d), few keratohyaline granules (blue arrow head) (2;c), edema of the underlying C.T. (stars) (2;a,b,c), dilated and congested blood vessels (red arrows) (2;b,c) (H&E: (a) 400X, (b) 400X, (c) 400X, (d) 1000X)

and pleomorphism (Fig. 2;d). Occasionally, few and scattered keratohyaline granules were noticed through the granular cell layer of some samples (Fig. 2;c). On the other hand, the basement membrane was obviously destructed with complete absence of the epithelial rete pegs. The lamina propria revealed absence of compactness, edema and severe degeneration together with dispersed, few fibroblasts and loosely arranged collagen fibers (Figs. 2;a,b,c). Dilated and congested blood vessels were also noticed within the connective tissue stroma (Figs. 2;b,c).

MTX and ginger treated group (group III)

The pathological changes of the MTX and ginger group were markedly attenuated and showed more or less normal appearance when compared to MTX treated rats. The epithelium apparently regained its normal thickness and appeared intact and continuous with uniform keratinous layer. The epithelial strata showed marked improvement of the signs of degeneration restoring the normal architecture of its cells. However, very few cells appeared with minimal cytoplasmic vacuolation and pyknotic nuclei. Numerous basophilic keratohyaline granules were identified within the granular cell layer. Again, the rete pegs displayed nearly normal configuration as that observed in group I. To certain extent, the underlying connective tissue exhibited better architecture with interlacing collagen fibers and numerous basophilic fibroblasts. However, few edematous areas could still be recognized (Fig. 3).

Molecular results

The quality of the DNA extracted from the buccal mucosa was represented by running electrophoresis on 0.8% agarose gel.

For each group six random primers were detected for DNA amplification using template DNA. All of them were able to generate reproducible amplification products with the tested primers.

Our study aimed to investigate the difference of genomic DNA between the control group (C),
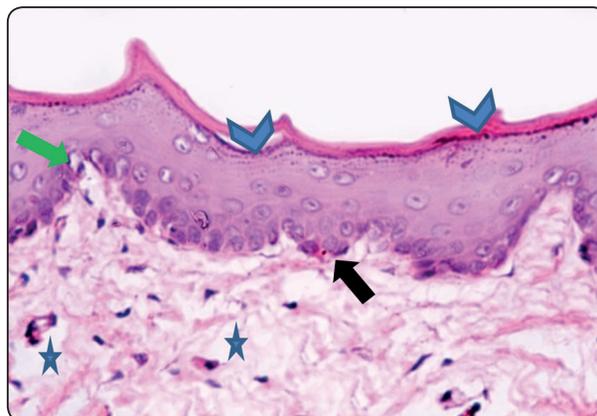

Fig. (3) Photomicrograph of rat buccal mucosa of group III showing apparent normal architecture of the buccal mucosa, cytoplasmic vacuolation (green arrow), numerous keratohyaline granules (blue arrow heads), short & broad epithelial rete pegs (black arrow), few edematous areas (stars) (H&E: 400X)

MTX treated group (T) and MTX and ginger treated group (R).

PCR product analysis by gel electrophoresis of genomic DNA isolated from the buccal mucosa of untreated and treated rats showed that each one of the six primers gave different numbers of band patterns as follows:

IS1 primer: (Figure 4 & table 2)

➢ *Control group(C)*

IS1 primer gave 11 bands in this group.

➢ *MTX treated group (T)*

In rats treated with MTX, the IS1 primer gave 9 bands suggesting that the quantitative changes showed obvious decrease in DNA relative concentration when compared to the control group.

➢ *MTX and ginger treated group (R)*

In MTX and ginger treated group, the IS1 primer gave 10 bands. Quantitative changes showed some decrease in DNA relative concentration when compared to the control group.
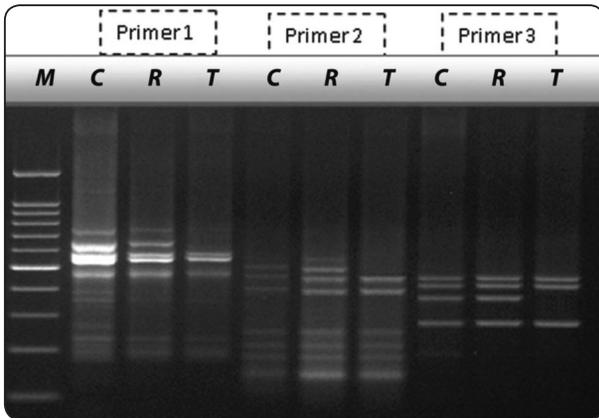
Fig. (4) An image showing PCR product by gel electrophoresis of genomic DNA of different groups using IS1, IS2 & IS3 primers.

TABLE (2) Analysis of the presence or absence of DNA bands of different groups using IS1 primer.

MW	C	R	T
1200	1	0	0
720	1	1	1
600	1	1	0
550	1	1	1
460	1	1	1
400	1	1	1
350	1	1	1
330	1	1	1
250	1	1	1
200	1	1	1
190	1	1	1
total	11	10	9

IS2 primer: (Figure 4 & table 3)

➢ *Control group(C)*

In the control group IS2 primer gave 8 bands.

➢ *MTX treated group (T)*

In rats treated with MTX, the IS2 primer gave

6 bands. The quantitative changes showed marked decrease in DNA relative concentration when compared to the control group.

➢ *MTX and ginger treated group (R)*

The IS2 primer in this group gave 8 bands. Quantitative changes showed similar DNA relative concentration in comparison to the control group.

TABLE (3) Analysis of the presence or absence of DNA bands of different groups using IS2 primer.

MW	C	R	T
600	1	1	0
550	1	1	0
460	1	1	1
400	1	1	1
260	1	1	1
240	1	1	1
200	1	1	1
150	1	1	1
total	8	8	6

IS3 primer: (Figure 4 & table 4)

➢ *Control group(C)*

In rats of the control group, IS3 primer gave 5 bands.

➢ *MTX treated group (T)*

In case of rats treated with MTX, the IS3 primer gave 3 bands indicating that the quantitative changes presented clear decrease in DNA relative concentration in comparison to the control group.

➢ *MTX and ginger treated group (R)*

IS3 primer in this group gave 4 bands. Quantitative changes showed some decrease in DNA relative concentration when compared to the control group.

TABLE (4) Analysis of the presence or absence of DNA bands of different groups using IS3 primer.

MW	C	R	T
480	1	1	1
440	1	1	1
350	1	1	0
270	1	1	1
160	1	0	0
total	5	4	3

IS4 primer: (Figure 5 & table 5)

➢ **Control group(C)**

In control group, IS4 primer gave 14 bands.

➢ **MTX treated group (T)**

In case of rats treated with MTX, the IS4 primer gave 11 bands. This presented marked decrease in DNA relative concentration compared to the control group.

➢ **MTX and ginger treated group (R)**

IS4 primer in this group gave 13 bands. Some decrease in DNA relative concentration was detected when compared to the control group.
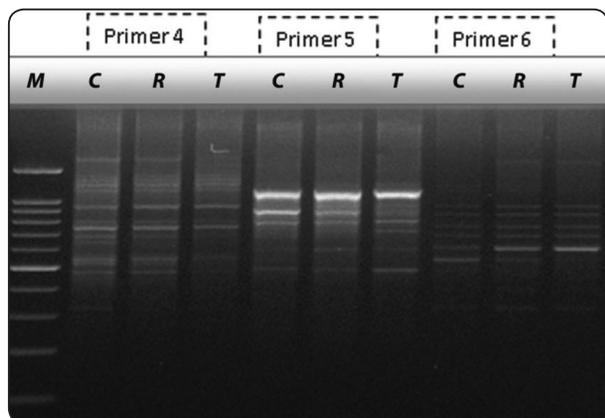

Fig. (5) An image showing PCR product by gel electrophoresis of genomic DNA of different groups using IS4, IS5 & IS6 primers.

TABLE (5) Analysis of the presence or absence of DNA bands of different groups using IS4 primer.

MW	C	R	T
1800	1	1	0
1500	1	1	1
1400	1	1	1
1200	1	1	1
1100	1	1	1
950	1	1	1
800	1	1	1
750	1	1	1
700	1	1	1
650	1	1	1
550	1	1	1
520	1	1	0
480	1	1	1
320	1	0	0
total	14	13	11

IS5 & IS6 primers: (Figure 5 & tables 6 & 7)

➢ **Control group(C), MTX treated group (T) and MTX and ginger treated group (R)**

IS5 & IS6 primers in untreated and treated groups gave 6 & 7 bands respectively.

TABLE (6) Analysis of the presence or absence of DNA bands of different groups using IS5 primer.

MW	C	R	T
1100	1	1	1
900	1	1	1
800	1	1	1
750	1	1	1
600	1	1	1
450	1	1	1
total	6	6	6

TABLE (7) Analysis of the presence or absence of DNA bands of different groups using IS6 primer.

MW	C	R	T
1050	1	1	1
950	1	1	1
850	1	1	1
750	1	1	1
620	1	1	1
550	1	1	1
300	1	1	1
total	7	7	7

Data concerning the matrix of similarity index were studied among the untreated control group (C) and treated groups (R & T) and represented in table 8. The matrix of similarity index between the control group (C) and the MTX treated group (T) was 87.4%, while that between the control and the MTX and ginger group (R) was 94.3%. These results presented marked decrease in DNA relative concentration in (T) group when compared to the control one, while less obvious decrease in DNA relative concentration was detected in (R) group.

TABLE (8) The matrix of similarity index among the three studied groups (C, R & T).

	C	R	T
C	100		
R	94.3	100	
T	87.4	91.5	100

The correlation between different numbers of band patterns detected by the six primers and the control (C), MTX (T) and MTX and ginger (R) groups were demonstrated in graph 1.
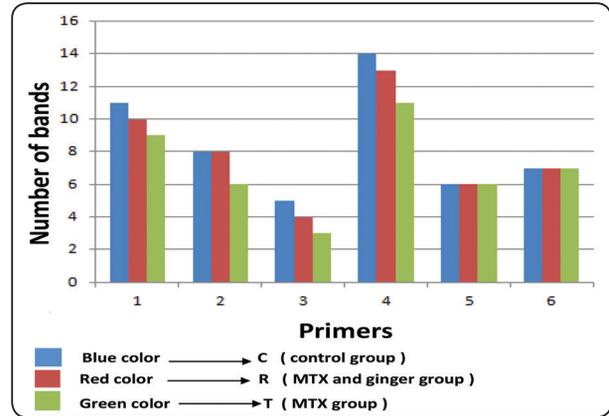
GRAPH (1) Different numbers of band patterns detected by the six primers among the three studied groups (C, R & T).

DISCUSSION

The present study aimed to show the histopathological and molecular changes of the rats' buccal mucosa post-application of methotrexate, and to evaluate the possible protective effect of ginger against methotrexate induced toxicity.

Methotrexate (MTX) is a powerful chemotherapeutic agent. However, it is of limited use as it is reported to cause toxic effects on various organs such as liver, kidney, testis, and heart **(Ciftci et al, 2011)** [19].

As all chemotherapeutic agents, MTX was found to damage neoplastic and non-neoplastic rapidly dividing cells in the body as the gastrointestinal epithelium, including the buccal epithelial cells **(Munaretto et al, 2011 and Akacha et al, 2015)** [8,20]. The mucosa is highly sensitive to cytotoxic drugs due to their high rate of cell proliferation and epithelial renewal **(Al-Refai et al, 2014)** [21].

The dose of MTX was administered during three consecutive days. This schedule was found to diminish the toxicity of the drug leading to reduction of possible death among the experimental animals **(Vanderhoof et al, 1990)** [22].

In the present work, H & E stained sections from MTX treated rats revealed apparent atrophy of the epithelial lining with areas of desquamated epithelium and overlying keratin. Meanwhile, few and sparse keratohyaline granules were detected resulting in the formation of thin or detached keratin layer. This data was coincident with **Munaretto et al, 2011** [8] who reported that thickness of the epithelial layers of the ventral surface of tongue in MTX treated mice was significantly lower than in control samples. Also, villous shortening and epithelial atrophy of the small intestine were persistent features of methotrexate treated rats (**Koppelmann et al, 2012**) [23], while areas of epithelial desquamation were reported by **Jahovic et al, 2004** [24]. Possible explanation suggested that this finding was due to the cytotoxic effect of MTX and to its interference of cell proliferation (**Yamamoto et al, 2013**) [25]. **In 2009, Lotfy and Zayed** [26] postulated that the direct suppression of DNA replication and cellular proliferation caused by MTX result in decrease in basal epithelial cells renewal, leading to atrophy. It was noted that atrophy and possible epithelial ulceration mostly occur in experimental animals from day zero to the fifth day following chemotherapeutic drugs application (**Berger and Clark-Snow, 2001**) [27].

Major histopathological changes were obviously detected in group II treated rats denoting signs of degeneration of the buccal mucosa. These changes included loss of normal architecture of the epithelial layers together with existence of pleomorphic cells and cytoplasmic vacuolation. Apoptotic cells showing pyknotic and fragmented nuclei were also spotted. These forgoing observations were mostly in agreement with **Ali et al, 2014 and Çakır et al, 2015** [28, 29], who demonstrated cellular pleomorphism and vacuolation together with apoptotic like lesions of heterochromatic, pyknotic and fragmented nuclei in MTX treated rats jejunum and liver respectively. Meanwhile, similar to our results, **Antar et al, 2005** [30] documented loss of villus architecture in the crypts of the small intestine of MTX subjected rats.

Furthermore, in the herein study the lamina propria revealed edema and severe degeneration together with few fibroblasts and loosely arranged collagen fibers. These findings go hand in hand with **Sritulasi et al, 2010** [31] who proved loss of compactness and edema in uterine stroma of MTX treated ovariectomized rats. Moreover, **Chen et al, 2013** [6] reported intestinal mucosal damage and obvious edema in MTX treated mice.

Previous results may support the concept that MTX treatment results in raise of lipid peroxidation and decrease in reduced glutathione which support oxidative stress. Thus, cause suppression of the effect of the antioxidant enzymes and consequently, makes the cells more sensitive to reactive oxygen species (ROS) (**Abd-Allah and Sharaf El-Din, 2013**) [2]. Other investigators clarified that free radicals bind to polyunsaturated fatty acids in the endoplasmic reticulum and to membrane lipids which stimulate cell destruction (**Naik, S. and Panda, 2007**) [32]. Therefore, lipid peroxidation can be a vital cause of cell membrane damage and may be responsible for the MTX-mediated tissue injury (**Akacha et al, 2015**) [20].

The histopathological results support the DNA molecular results which showed marked decrease in DNA relative concentration and matrix of similarity index in MTX treated group when compared to the control one in the first four studied primers. A suggestion could be interpreted supporting that the ROS generated by MTX have deleterious effects on the DNA of epithelial cells (**Shih et al, 2003**) [33]. MTX prevents the synthesis of DNA by binding to the enzyme dihydrofolate reductase (**Sukhotnik et al, 2014**) [34]. This induces blocking of synthesis of the nucleoside thymidine and purine, required for DNA synthesis. So, MTX was found to cause toxic effects by arresting the growth and proliferation of neoplastic and some non- neoplastic cells (**Sritulasi et al, 2010**) [31]. Also, it triggers alterations in gene expression and subsequently can cause cell death. An interesting finding is that MTX not only promotes apoptosis throughout the S-phase of cell

cycle during DNA replication, but it can also bring about apoptotic cell death after mitosis, in which DNA synthesis does not occur (**Neradil et al, 2012**) [35]. Meanwhile, **Imani and Ainehchi, 2014** [4] added that MTX creates covalent bonds in nucleophilic position of DNA proteins and strands. Hence, results in disruption of DNA strands, discontinuing DNA synthesis and formation of small nuclei and finally, cell death.

A persistent feature in group II treated animals was the existence of dilated and congested blood vessels within the connective tissue stroma. These data were parallel to that reported by **Patel et al, 2014 and Çakır et al, 2015** [36, 29] in liver sections of MTX treated rats. Possible explanation was introduced by **Cawley and Benson, 2005** [37] who concluded that in the initial phase of mucositis, the epithelial, endothelial, and connective tissue cells in the buccal mucosa liberate free radicals and pro-inflammatory cytokines, such as interleukin-1B, prostaglandins, and Tumor Necrosis Factor (TNF). These mediators increase vascular permeability and angiogenesis, resulting in enhancement of cytotoxic drug uptake into the oral mucosa.

Recently, attention has been drawn on the protective effect of natural plant derived compounds against toxic drugs. In this work, we chose ginger as one of the available medicinal plants and studied its ameliorative effect on methotrexate toxicity. Ginger is a safe, non-toxic herbal medication with undetectable side effects (**Badreldin et al, 2008**) [38]. It is used as an alternative and supportive therapy showing a promising effect (**Sabik and Abd El-Rahman, 2009**) [39].

In the present study we administrated ginger before and during MTX treatment. **Sabik and Abd El-Rahman, 2009** [39] verified that application of ginger before and during cyclophosphamide treatment (chemotherapeutic drug) reinforce the antioxidant system, abolish oxidative reaction and act against chemotherapy induced toxicity. Another study postulated that ginger extract prior and co-administration with cisplatin (chemotherapeutic drug) offered almost complete protection in terms of organs histological changes and plasma biochemical changes (**Attyah, and Ismail, 2012**) [15]. Furthermore, **Abd-Allah and Sharaf El-Din, 2013** [2] reported a protective effect of pre and post ginger treatment against intestinal damage induced by MTX in rats.

Histological results of MTX and ginger treated group showed marked amelioration of the architecture and all degenerative changes manifested by almost normal appearance of the buccal mucosa. These results were coincident with **Sabik and Abd El-Rahman, 2009** [39] who proved that pre and co-treatment of ginger reduced degenerative changes of prostatic and seminal vesicle epithelium induced by cyclophosphamide. These degenerative changes included epithelial desquamation and disorganization of epithelium, vacuolar degeneration, pyknosis, nuclear fragmentation and pleomorphism. Similar findings were obtained by **Attyah, and Ismail, 2012** [15] who postulated that no significant degenerative changes of hepatocytes could be monitored when ginger extract was used with cisplatin.

On the basis of these available results, some suggestions could be interpreted supporting that ginger is a powerful antioxidant agent which is capable of reducing the toxicity of many anticancer drugs (**Attyah, and Ismail, 2012**) [15]. Active phenolic ingredients of ginger (e.g., zingerone, gingerdiol, zingibrene, gingerols and shogaols) have been assigned for its antioxidant activity. In addition, ginger has been shown to decrease lipid peroxidation, raise the level of antioxidant enzymes (i.e., superoxide dismutase and catalase), plus acting as a free radical scavenger **Abd-Allah and Sharaf El-Din, 2013** [2]. This may explain the protective effect of ginger in the attenuation of the previously mentioned deleterious effects of MTX, and preservation of the structural integrity of the buccal mucosa.

DNA molecular results revealed less obvious decrease in DNA relative concentration and matrix of similarity index in MTX and ginger treated group compared to the control one within the first four studied primers. These results agree with several studies that show the protective ability of ginger against induced DNA damage. In this regard, **Lu et al, 2003** [40] proposed that ginger caused significant decrease in the degree of DNA destruction induced by H_2O_2 in rabbit hepatocyte in vitro when compared with the control group. Accordingly, this is further supported by **Ajith, 2010** [41] who reported that ginger extract could partially improve the DNA damage induced by H_2O_2 in rat liver. It was also proved that ginger increased the comet ratios indicating decrease in the extent of DNA damage when tested for its protecting effect against benzo(a)pyrine induced DNA injury of human blood lymphocytes (**Nirmala et al, 2007**) [42]. Other investigators showed that the frequencies of chromosomal aberrations were reduced and were not significantly different from control when ginger was supplemented with the anti-cancer drug Taxol in bone marrow cells of mice (**AL-Sharif, 2011**) [43]. Moreover, **Jayakumar and Kanthimathi, 2012** [44] found that pretreatment of murine fibroblasts with ginger in cell culture before exposure to H_2O_2 protected against 68% of DNA damage.

Possible explanation documented that beside the endogenous antioxidant mechanisms, a diet rich in antioxidant foodstuffs is able to protect DNA and to enhance the resistant effect against oxidative damage. Ginger, one of the plant derived products, is found to have cyto-protective as well as DNA protective properties (**Bisht et al 2010**) [45].

In conclusion, experimental data of our work provided evidence that MTX treatment is associated with induction of oxidative stress. Histopathological and molecular evaluations showed that pre and co administration of ginger protected and ameliorated most of the damage caused by MTX in the buccal mucosa structure and DNA, proving a positive role for medicinal herbs against chemotherapy induced toxicities.

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